Readapting the Adaptive Immune Response – Therapeutic Strategies for Atherosclerosis

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Abstract

Cardiovascular diseases remain a major global health issue, with the development of atherosclerosis a major underlying cause. Our treatment of cardiovascular disease has improved greatly over the past 3 decades, but significant need still remains to reduce disease burden. Current priorities include reducing atherosclerosis advancement to clinically significant stages, and preventing plaque rupture or erosion. Inflammation and involvement of the adaptive immune system influences all these aspects and therefore is one focus for future therapeutic development. The atherosclerotic vascular wall is now recognised to be invaded from both sides (arterial lumen and adventitia), for better or worse, by the adaptive immune system. Atherosclerosis is also impacted at multiple stages by adaptive immune responses, overall providing multiple opportunities to target these responses to reduce disease progression. Protective influences that may be defective in diseased individuals include humoral responses to modified LDL and regulatory T cell responses. There are multiple strategies in development to boost these pathways in humans, including vaccine-based therapies. The impact of various existing adaptive immune targeting therapies, such as blocking critical costimulatory pathways or B cell depletion, on cardiovascular disease are beginning to emerge with important consequences for both autoimmune disease patients and the potential for wider use of such therapies. Entering the translation phase for adaptive immune targeting therapies is an exciting and promising prospect.

Tables of Links

TARGETS	
Other protein targets[a]	**Enzymes**[b]
CD20	HMG CoA Reductase
CD80	
CD86	
PD-1	

LIGANDS
BAFF

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander et al., 2015).*

Abbreviations

Acute coronary syndrome (ACS), adventitial tertiary lymphoid organs (ATLO), antigen presenting cells (APCs), cardiovascular disease (CVD), coronary artery disease (CAD), heat shock protein (hsp), human leukocyte antigens (HLA), interferon (IFN), interleukin (IL), low density lipoprotein (LDL), major histocompatibility complexes (MHC), malondialdehyde (MDA), myocardial infarction (MI), regulatory T cells (Treg), phosphorylcholine (PC), single nucleotide polymorphisms (SNPs).

Introduction

Cardiovascular diseases remain a major global health issue. A major underlying cause of myocardial infarction and stroke is atherosclerosis, the chronic inflammatory response to injury in the vascular wall of major arteries at specific sites of susceptibility (Lusis, 2000). Atherosclerotic plaque is initiated by accumulation and modification of low density lipoprotein (LDL) cholesterol below the endothelial cell layer. Subsequent progression, which may occur discontinuously over decades, include inflammatory activation, infiltration and proliferation of both immune cells and local vascular smooth muscle cells, and matrix deposition (Lichtman *et al*., 2013; Bentzon *et al*., 2014). The balance between accumulation of lipid-filled foam cells, lack of clearance of apoptotic or necrotic cells, deposition of extracellular matrix and immune cell polarization intrinsically determine the growth and stability of the plaque. Extrinsically, many factors impact on atherosclerosis, but perhaps none more prominently than circulating LDL levels. This is emphasised by the effectiveness of statins in reducing the risk of cardiovascular events (Collins *et al*., 2016). Plaque rupture, more specifically of the fibrous cap, is the most common cause of acute events. For example, rupture underlies 73% of fatal coronary thrombi (Falk *et al*., 2013), with plaque erosion the most common alternative event. In the era of cholesterol-lowering drugs (e.g. statins), there is a trend towards diminishing occurrence of plaque rupture (Libby and Pasterkamp, 2015). Our treatment of cardiovascular disease has improved greatly over the past 3 decades. For example, the UK has seen a 70% drop in age-adjusted cardiovascular mortality since 1979. However, over the same period, prevalence has remained stable (Bhatnagar *et al*., 2016). Current priorities include identifying at risk patients in more precise and timely ways, reducing atherosclerosis advancement to clinically significant stages, and preventing plaque rupture or erosion. Inflammation and recruitment of the adaptive immune system may be important in all of these aspects and is one focus for future therapeutic development.

Adaptive immune cells, T cells and B cells, have a unique ability to create clonally distinct antigen receptors through somatic rearrangement of genomic DNA, and (in B cells) through further mutation. This creates an antigen receptor repertoire of near-global specificity; B cells recognise diverse epitopes within whole molecules, T cells recognise peptides presented within major histocompatibility complexes (MHC) on antigen presenting cells (APCs). The MHC Class I (for $CD8^+$ T cells) and MHC Class II (for $CD4^+$) are named human leukocyte antigens (HLA) in humans. Normally, multiple layers of tolerance mechanisms limit a significant involvement of T cells in non-infectious inflammation. These include deletion of

autoreactive clones during thymic development, anergy through overstimulation, or dominance of regulatory T cells (Treg) that curtail initial T cell responses. T cell clones may overcome these mechanisms via defective negative selection, continued endurance of otherwise anergic T cells, or failed Treg control. T cell specificity, APC stimulatory capacity and cytokine milieu are key parameters dictating these changes. For example, $CD4^+$ T cells are polarized to functional subtypes including Th1, Th2, Th17, follicular helper (Tfh) or Treg. B cells are also tamed by tolerance checkpoints; in addition the most potent, pro-inflammatory responses are T cell-dependent, providing another level of control.

The atherosclerotic vascular wall is invaded from both sides (arterial lumen and adventitia), for better or worse, by the adaptive immune system. We now understand a great deal about the impact of adaptive immune responses at different stages of cardiovascular disease, with both pathogenic and protective influences delineated. We are now in a phase of translating these findings to the clinic and trialling therapeutic strategies in patients. Inflammation may represent a significant proportion of the residual risk for cardiovascular disease remaining in patients on current therapies. If adaptive immune responses occur early on during disease development or before therapies are initiated, then despite reduction in risk factors (LDL cholesterol, innate cytokines), the memory compartment may endure and continue to produce pathogenic effectors. Strategies targeting adaptive immune components could therefore provide key future therapies to mitigate residual risk in patients. We will first discuss in turn analysis of human atherosclerotic patient samples, and functional studies in experimental models. We then summarize some of the therapeutic strategies targeting adaptive immunity currently being considered.

Atherosclerosis and the Adaptive Immune System in Patients

Local Evidence - T cells

The most clinically relevant sites of atherosclerotic plaque development are the coronary and carotid arteries, with the aorta and its major branches also important sites in both humans and experimental models. Recent evidence significantly strengthened the association of adaptive immune infiltration with adverse outcomes or unstable status of plaques. T cells are found within coronary atherosclerotic plaques at multiple stages of disease progression (Boyle, 1997; Liuzzo *et al*., 2000; Watanabe *et al*., 2007; Kortelainen and Porvari, 2014; Otsuka *et al*., 2015). Otsuka *et al* (2015) recently found $CD3^+$ T cells in early lesions classified as pathological intimal thickening, but only once $CD68^+$ cells (macrophages) were also present.

T cells were then continuously present in later stages (fibroathermomas) of coronary arteries. Many studies have also detected T cells within carotid plaques (Kleindienst *et al*., 1993; Palma *et al*., 2006; de Boer *et al*., 2007; Chen *et al*., 2009; Erbel *et al*., 2011; Dietel *et al*., 2013; Rohm *et al*., 2015). Plaques of otherwise similar status in older patients contain higher levels of infiltrating T cells (Najib *et al*., 2012). Both macrophages and T cells are present in higher proportions in the fibrous cap of ruptured plaques (Falk, 1983; Lendon *et al*., 1991; van der Wal *et al*., 1994; Boyle, 1997; Otsuka *et al*., 2015). Erbel *et al* (2011) recently showed that T cell infiltration was significantly associated with ischemia. In the aorta, an increased T cell response in both plaque and adventitia peaks in correlation with plaque instability, with reduced T cell infiltration in healed plaques (van Dijk *et al*., 2015). Increased infiltration of effector T helper cells rather than Treg cells is a hallmark of unstable compared to stable carotid artery plaques (Dietel *et al*., 2013; Rohm *et al*., 2015). Altogether, T cell responses are involved in both early plaque progression and late stage degradation of fibrous caps, but the links and similarities between these responses are still unclear. In addition to T cells themselves, the molecules able to locally activate T cells are abundantly expressed by APCs in atherosclerotic plaque, for example HLA (Jonasson *et al*., 1985; Xu *et al*., 1990) and costimulatory molecules (de Boer *et al*., 1997). In the adventitia of atherosclerotic vessels, adaptive immune cell infiltration is commonly detected, and can advance to formation of organized tertiary lymphoid organs (Boyle, 1997; Houtkamp *et al*., 2001; Clement *et al*., 2014). $CD8^+$ T cells are quite common in advanced human atheroma and are found both in plaque and adventitia (Zhou *et al*., 1996; van Dijk *et al*., 2015). Early studies suggested less involvement of $CD8^+$ compared to $CD4^+$ T cells (Zhou *et al*., 1996), but the potential importance of $CD8^+$ T cells has now been revisited (see below).

Initial studies more than 20 years ago demonstrated human plaque T cells respond to oxidized LDL (oxLDL)-derived peptides presented on HLA molecules (Stemme *et al*., 1995) and subsequently to heat shock protein (hsp)60 (Benagiano *et al*., 2005; Almanzar *et al*., 2012). In the study by Stemme *et al* (1995), isolation and stimulation of T cell clones from atherosclerotic plaques determined a significant proportion proliferated in response to oxLDL and were Th1 polarized, producing interferon (IFN)-γ. This seminal study established the paradigm that oxLDL represents a neo-self antigen responsible for activating and recruiting a T cell-driven autoimmune response against the artery wall. Benagiano *et al*., (2003) similarly found a predominance of $CD4^+$ Th1 polarized clones. An alternative T cell antigen is hsp60, which is upregulated on inflamed endothelial cells. Hsp60 bears close similarity with

bacterial hsp (GroEL), so T cells initially activated by bacterial antigens could also regulate atherosclerosis (Mosorin *et al*., 2000; Benagiano *et al*., 2005).

Local Evidence – B cells and Antibodies

B cells are very rare within atherosclerotic plaques themselves, but are more often found in adventitial inflammatory cell infiltrates (Boyle, 1997; van Dijk *et al*., 2015). PCR approaches have been successful in detecting the presence of B cells in the plaque and/or adventitia of coronary and carotid plaques (Burioni *et al*., 2009; Hamze *et al*., 2013). These studies showed the presence of class-switched, somatically mutated B cell receptor genes and the presence of limited but expanded clones. In addition, IgG and IgA expressing cells were most common in one study (Hamze *et al*., 2013). These studies confirm the presence of an active ongoing B cell humoral response in advanced stages of atherosclerosis. Interestingly, Hamze *et al* (2013) concluded that adventitial and plaque B cell clones were distinct and did not represent a directly linked response. How systemic responses that originate in spleen or draining lymph nodes relate in terms of antigen specificity and functional effects to local plaque and adventitial responses is currently unknown.

Antibodies, however, abundantly bind within atherosclerotic plaques. The assumed major specificity of these antibodies is modified lipids, however many other autoantigens may be targeted (Merched *et al*., 2016). Modified lipid epitopes are certainly present, as exemplified by anti-phosphorylcholine (PC; clone E06) staining in plaques (van Dijk *et al*., 2012). As in other autoimmune diseases, a major target could also be extracellular matrix or nuclear antigens. High levels of anti-nuclear antibodies are not a prominent feature of coronary artery disease (CAD) patients unless autoimmunity is present. Nevertheless, one study associated enhanced circulating anti-nuclear antibodies in symptomatic compared to asymptomatic carotid artery stenosis patients (Döring *et al*., 2012). IgE is also found in human carotid atherosclerotic plaques in areas of high CD68 expression and $TUNEL^+$ apoptotic cells (Wang *et al*., 2011). In terms of antibody effector pathways, the classical complement pathway (primarily initiated by immune complexes) appears to be active in human atherosclerotic plaques (Kimoto *et al*., 1996; Oksjoki *et al*., 2007). Antibodies also influence cellular functions via ligation of cell surface Fc receptors. There is a family of Fcγ receptors (I – IV) for IgG, FcεR (CD23) for IgE, FcμR (Toso) for IgM and the neonatal Fc receptor (FcRn), which is important in IgG recycling (Smith and Clatworthy, 2010). Macrophages within atherosclerotic plaques are influenced by IgG immune complexes via Fcγ receptors and

vulnerable plaques exhibit enhanced levels of FcγR and downstream signalling molecules (Lennartz *et al*., 2011). FcγRIII (CD16) is an important marker of non-classical patrolling monocytes, compared to classical inflammatory monocytes that express high levels of the toll-like receptor 4 co-receptor CD14. Endothelial cells and vascular smooth muscle cells also express FcγR (Tanigaki *et al*., 2015). In summary, there is evidence for an adaptive immune cell response ongoing within atherosclerosis, as well as the presence of upstream activating and downstream effector pathways. The levels of these responses correlate closely with clinically relevant plaque status.

Systemic, Genetic and Risk factor-related Evidence

The analysis of circulating T cell subsets has also informed us about potential roles in cardiovascular disease (reviewed in Ammirati *et al*., 2015). As in atherosclerotic plaques, T cells reactive to oxLDL are present in the blood of cardiovascular disease patients (Frostegård *et al*., 1992). Various functional T cell subsets have been associated with CAD patients. Ammirati *et al* found a positive correlation between effector memory T cells and cardiovascular disease (Ammirati *et al*., 2012). In contrast, regulatory T cell levels are reduced in CAD patients (Mor *et al*., 2006; Ji *et al*., 2009; Wigren *et al*., 2012). A prominent feature of the cardiovascular patients' T cell system is the expansion of an otherwise rare population of $CD4^+$ $CD28^-$ T cells. This population increases with chronic inflammation, produces IFN-γ and acquires cytotoxic functionality not normally associated with $CD4^+$ T cells (Dumitriu *et al*., 2012; Téo *et al*., 2013). One study found that $CD4^+$ $CD28^-$ cells responded to hsp60 (Zal *et al*., 2004). Chronic low grade inflammation may promote re-stimulation of these cells, leading to loss of CD28 (Bobryshev, 2010; Chistiakov *et al*., 2016), as well as resistance to apoptosis (Kovalcsik *et al*., 2014). Acute coronary syndrome (ACS) patients have a reduced T cell repertoire compared to healthy controls, suggesting accelerated ageing or systemic expansion of certain, perhaps autoreactive, clones (Klingenberg *et al*., 2014).

A large number of studies have correlated modified lipid-binding antibody levels to various aspects of cardiovascular disease, as reviewed in more detail elsewhere (Carbone *et al*., 2013). A consistent finding is the inverse correlation of cardiovascular disease (CVD) with anti-oxidized lipid IgM antibodies (Karvonen *et al*., 2003). These ubiquitous and common antibodies may be important in limiting the accumulation of oxidized lipid debris and preventing foam cell formation. An alternative possibility could involve acting on PC and

malondialdehyde (MDA)-positive bacteria. Low levels of anti-PC IgM can predict mortality (Carrero *et al*., 2009) whereas high levels are associated with slower atherosclerosis progression (Su *et al*., 2005; Ravandi *et al*., 2011). Single nucleotide polymorphisms (SNPs) close to genes encoding TNFSF13 (APRIL), a known plasma cell survival factor, and its receptor TNFRSF13B (TACI) were associated with IgM levels in humans (Osman *et al*., 2012). IgG antibodies to the same epitopes have more inconsistent correlations. For example, Lehtimäki *et al*., (1999) found a significant positive association with atherosclerosis, whereas in a prospective observational study, Wilson *et al*., (2012) found no impact on risk. Another recent study demonstrated high levels of antibodies binding ApoB100 in LDL are associated with reduced CVD (Björkbacka *et al*., 2016). Khamis *et al*., (2016) recently found total IgG levels correlated with CVD and adjusting for total levels negated the correlation of oxLDL-specific IgG. Increased serum IgE levels have been associated with enhanced risk of CAD, and in particular plaque stability (Kounis and Hahalis, 2016). In addition, IgE as well as eosinophil and mast cell responses occur as part of immune responses in acute coronary syndromes (Kritikou *et al*., 2016).

A recent study used genomics data to determine that 2 B cell regulatory genes, TNFSF13B (B cell activating factor; BAFF) and SPIB, were the core regulatory factors in a module of genes co-regulated in non-CVD subjects but deregulated in CVD patients (Huan *et al*., 2013). Although neither BAFF nor SPIB are entirely B cell specific, this study supports the idea that significant changes in B cell responses are associated with CVD. A major function for interleukin (IL)5, and one of many functions of IL6, is to promote B cell humoral responses, and both IL5 and IL6R are candidate target genes of 2 genomic loci associated with CVD through GWAS analysis (Nikpay *et al.,* 2015), as is the chromosome 6p21 region, which contains the Class I HLA locus (Davies *et al*., 2012). Soluble IL2Rα, a decoy receptor for lymphocyte growth factor IL2, associates with CVD even after adjustment for other risk factors; genetic polymorphisms at chromosome 10p15-14 influence the level of sIL2Rα, but do not directly link to increased incidence of CVD (Durda *et al*., 2015). The chemokine CXCL13 was recently shown to correlate closely with germinal center B cell responses in humans (Havenar-Daughton *et al*., 2016), although it does have innate immune functions as well. Higher CXCL13 levels were found in cardiovascular disease patients, particularly those with symptomatic status (Smedbakken *et al*., 2012). This systemic data supports the histology data showing adaptive immune responses correlating closely with plaque status (van Dijk *et al*., 2015; see above).

Many central risk factors for cardiovascular disease and atherosclerosis may also elicit adaptive immune responses. The immune system declines with age (reviewed in Linterman, 2014). However, a decrease in the strength of responses to infections or vaccinations is matched with a paradoxical increase in autoimmune propensity. The roles of adaptive immune responses in hypertension, obesity and type II diabetes have mainly been addressed in animal models and are considered briefly below.

Atherosclerosis and the Adaptive Immune System in Experimental Models

The functional importance of adaptive immunity in atherosclerosis is demonstrated by a plethora of *in vivo* studies in animal models, most extensively in the complementary mouse models of hypercholesterolemia, *Apoe*$^{-/-}$ and *Ldlr*$^{-/-}$ mice, but also in hypercholesteraemic rabbits. A limit to translating experimental findings into therapies is differences between the murine and human immune systems. Examples of significant differences include distinct functional properties of antibody isotypes and Fc receptor-dependent responses between mice and humans (Smith and Clatworthy, 2010) and the ability of mouse but not human thymus to maintain the naïve T cell pool (den Braber *et al*., 2012). A recent study suggested entirely different transcriptional profiles of mouse and human responses to a range of diseases (Seok *et al*., 2013), however this study was challenged soon after (Takao and Miyakawa, 2014). Accepting these caveats, major findings on the involvement of B cell and T cell responses in humans (as far as they are understood) are faithfully reproduced in mice. Several consistent findings allow us to assign important anti-atherogenic status to regulatory T cells and to natural IgM-producing B1 cells. Depletion of Tregs leads to increased atherosclerosis (Ait-Oufella *et al*., 2006; Gotsman *et al*., 2006). Interference with conventional dendritic cells also leads to reduced Treg levels and increased atherosclerosis (Choi *et al*., 2011; Subramanian *et al*., 2013). As atherosclerosis progresses, local Treg levels are reduced in *Ldlr*$^{-/-}$ mice (Maganto-García *et al*., 2011). This could potentially relate to their conversion to Il17^{+} or IFN-γ producing Th1-like cells (Butcher *et al*., 2016). Regulatory T cells dampen immune responses in multiple ways and can both prevent responses from taking off as well as accelerating the resolution phase. Regulatory T cells are more sensitive than effector or naïve T cells to IL2. Once Treg recognise antigen, they can repress APC by removing costimulatory molecules (e.g. through cytotoxic T lymphocyte antigen-4 (CTLA4)) and by secreting IL10 and transforming growth factor-β.

The protective role of B1 cells is supported by multiple studies in mouse models. The enhanced atherosclerosis in splenectomised mice is reversed by transfer of B1 but not B2 cells (Kyaw *et al*., 2011). Both B1a ($CD5^+$) and B1b ($CD5^-$) cells are capable of producing atheroprotective IgM (Binder *et al*., 2003; Rosenfeld *et al*., 2015). Unlike IgG antibodies, IgM does not bind to pro-inflammatory Fc receptors on macrophages and neutrophils, although it can be associated with promoting antigen presentation to T Cells. The oxidized epitopes targeted by atheroprotective IgM are found on various circulating particles including oxLDL, Lipoprotein(a), apoptotic cells and microparticles, all of which may also accumulate within atherosclerotic plaques (Tsiantoulas *et al*., 2015). It remains to be determined whether IgM removal of all or only particular carriers of these epitopes is important for atheroprotection. An atheroprotective clonotype of anti-PC IgM, T15/E06, is encoded by the V1 heavy chain gene. Although IgM encoded by this gene is important for protection from *Staphylococcus pneumoniae* infection, atherosclerosis was not affected and overall IgM reactive with PC was not significantly affected (Centa *et al*., 2015). As the most prominent antagonist to these protective pathways, Th1 type $CD4^+$ T cells promote atherosclerosis. Production of cytokines IFN-γ and IL12 are important mediators of this role (Davenport and Tipping, 2003; Buono *et al*., 2005). The IL1 family cytokine IL18 is also a strong signal for Th1 differentiation when in combination with IL12 or IL15 (Novick *et al*., 2013). In mice, IL18 promotes atherosclerosis (Mallat *et al*., 2001) and a recent paper showed it can act via both IL18 receptor and an alternative receptor, SLC12A3 (Wang *et al*., 2015). IFN-γ can activate macrophages towards a pro-inflammatory phenotype and also directly targets vascular cells. B cell production of granulocyte-monocyte-colony stimulating factor (Hilgendorf *et al*., 2014) and antigen presentation by plasmacytoid DCs (Sage *et al*., 2014) are upstream regulators of Th1-driven atherosclerosis.

Other lymphocyte subsets have less well defined roles, with studies in mice showing opposing effects on atherosclerosis (Legein *et al*., 2013; Witztum and Lichtman, 2014; Taleb *et al*., 2015). This can be down to differences in experimental design and conditions between labs as well as to the multifunctional roles of theses subsets. For example, B2 cells have been ascribed both pathogenic and protective roles based on different experimental approaches. B2 cell depletion leaving B1 cells intact leads to reduce atherosclerosis (Ait-Oufella *et al*., 2010; Kyaw *et al*., 2012; Sage *et al*., 2012), however adoptive transfer of purified B2 cells into B cell deficient (lacking both B1 and B2 cell) mice was either pro- or anti-atherogenic in 2 different studies (Kyaw *et al*., 2010; Doran *et al*., 2012). Autoimmune disease models

associated with B2 cell production of autoantibodies are generally associated with increased atherosclerosis (Gautier *et al*., 2007; Clement *et al*., 2014; Merched *et al*., 2016). Indirectly, knockout of pro-inflammatory IgG (Fcγ) receptors results in reduced atherosclerosis (Hernández-Vargas *et al*., 2006; Kelly *et al*., 2010), supporting a pathogenic role for autoantibodies. Although there is evidence of pathogenic influence, anti-oxLDL IgG is most likely protective in the same way as IgM of the same specificity. Treatment of atherosclerotic mice in a regression model (switched to chow from high-fat diet) with human monoclonal IgG1 to MDA-modified ApoB100 resulted in accelerated regression and 50% less atherosclerosis than the control group (Schiopu *et al*., 2007). Initial studies with mice lacking inhibitory FcγRIIb showed more atherosclerosis, however more recently the opposite effect was shown, with a mixed genetic background in the early studies a critical difference (Mendez-Fernandez *et al*., 2011; Harmon *et al*., 2014; Ng *et al*., 2015). Given the diverse subsets and response types of adaptive B2 cells, more models that can target specific functions or prevent antigen-specific responses are required to more accurately probe the potential roles for B2 cells. Several studies have suggested that $CD8^+$ T cells, not commonly found in mouse atherosclerotic plaques, do not play a significant role in atherosclerosis (Zhou *et al*., 1996; Kolbus *et al*., 2012), however $CD8^+$ T cells can promote atherosclerosis in some studies (Kyaw *et al*., 2013). A recent study demonstrated that in addition to their classical cytotoxic function, the lack of $CD8^+$ T cells significantly modulated monocyte production in the bone marrow (Cochain *et al*., 2015). Given the recent genetic association of cardiovascular disease with the $CD8^+$ T cell activating MHC Class I locus (Davies *et al*., 2012), further study of the role of $CD8^+$ T cells is certainly warranted.

An adventitial adaptive immune response in the descending aorta of *Apoe*$^{-/-}$ mice can occur quite early during experimental models (Galkina *et al*., 2006; Koltsova *et al*., 2012), and in old *Apoe*$^{-/-}$ mice, these responses become organized into adventitial tertiary lymphoid organs (ATLO) (Srikakulapu *et al*., 2016). A recent study used smooth muscle cell-specific deletion of lymphotoxin β receptor to prevent ATLO formation and found that this exacerbated atherosclerosis (Hu *et al*., 2015). Microvessels are abundantly present in atherosclerotic plaque (Collett and Canfield, 2005), responding to plaque hypoxia (Marsch *et al*., 2014). Such microvascular networks provide the possibility for direct interaction of adventitial and plaque immunity. Adventitial responses can alternatively have systemic consequences for cardiovascular disease. T cell responses in perivascular adipose tissue are essential for the development of experimental hypertension (Itani *et al*., 2016), although human evidence of

these links is sparse. In early atherosclerosis, dendritic cells scanning sites of atherosclerosis susceptibility were recently shown to exit back into the vessel lumen (intravasation) (Roufaiel *et al*., 2016), providing another potential route and suggesting systemic T cell recruitment.

Beyond atherosclerosis models, T cell and B cell responses also play prominent roles in co-morbidities for cardiovascular disease. Autoimmune driven arthritis and SLE have a clear adaptive immune component. Increasingly, models of type II diabetes reveal important potential roles for the adaptive immune system. Like in atherosclerosis, T cells that display an effector/memory phenotype, and are predominantly Th1-polarised, infiltrate obese adipose tissue and promote insulin resistance (reviewed in Majdoubi *et al*., 2016). B cell responses originating in adipose tissue may also be important in type II diabetes. For example, transfer of wildtype but not $MHCII^{-/-}$ B cells to B cell-deficient obese mice induced insulin resistance (Winer *et al*., 2011).

Therapeutic Strategies Targeting the Adaptive Immune System

Current therapies for cardiovascular disease designed to primarily target other disease processes may also act in part through actions on the adaptive immune system (reviewed in (Vré *et al*., 2011). The anti-inflammatory properties of cholesterol-lowering statins are well-known (Jain and Ridker, 2005) including direct potential effects on adaptive immune responses. For example, simvastatin reduces MHCII expression (Kwak *et al*., 2000) . The complex relationship between lymphocytes and systemic lipid metabolism is receiving more attention (Chyu *et al*., 2014; Sorci-Thomas and Thomas, 2016). Angiotensin-converting enzyme inhibitors used to lower blood pressure may also directly target T cells, which express Angiotensin type 1 receptor and play an important role in Angiotensin II-induced vascular pathology (Guzik *et al*., 2007).

Given the presence of both protective and pathogenic responses, therapeutic strategies designed against the adaptive immune system can be broadly divided into those attempting to boost protective pathways or those attempting to correct or inhibit pathogenic pathways. Strategies can also be global, by targeting costimulatory or cytokine signalling pathways, or antigen-specific. Costimulatory (or co-inhibitory) proteins form ligand-receptor pairs between interacting cell types and can dictate the outcomes for both cells and thus play a prominent role in guiding adaptive immune responses (den Haan *et al*., 2014). A prominent and well-studied costimulatory pairing is CD40-CD40 ligand, members of the TNF family. However, since this pathway has multiple impacts on atherosclerosis via important roles in

innate immune cell or platelets as well as the adaptive immune response, we do not discuss this pathway further here (Gerdes *et al*., 2016; Jansen *et al*., 2016). Methods to isolate locally induced (i.e. ATLO) responses from systemic responses are currently lacking in the clinical setting, but could form the basis for future strategies. Below, we discuss antigen-independent strategies to boost Treg-mediated protection or attenuate pathogenic responses. Antigen-specific immunization and tolerization strategies, targeting both T cells and B cells, are discussed finally. The targeted pathways of these strategies are illustrated in Figure 1. Most strategies outlined below could potentially attenuate both plaque progression and the risk of plaque rupture. Given the lack of widely used models of plaque rupture in mice, it is hard to assess impacts separately in experimental models. Immunization and tolerization strategies are the most likely strategy to be effective at earlier stages of disease.

Regulatory T cell Enhancing Therapies

Interference with CD80/86 – CD28 costimulation has shown to be an effective targeted immunosuppressive strategy, mimicking the effects of Tregs. Co-stimulation of $CD28^+$ T cells via CD80/86 on APCs drives atherosclerosis in mice (Buono *et al*., 2004; Matsumoto *et al*., 2016). Biological therapies based on CTLA4 are approved for rheumatoid arthritis and kidney transplantation. In mice, translational studies using CTLA-Ig (Abatacept) have shown promise. Ewing *et al* showed decreased intimal hyperplasia and inflammation with abatacept (Ewing *et al*., 2013), whereas Ma *et al* showed effective inhibition of homocysteine-accelerated atherosclerosis in *Apoe*$^{-/-}$ mice (Ma *et al*., 2013). IL2 triggers expansion and differentiation of both effector T cells and Treg. Treg have a significantly enhanced sensitivity to IL2 and this underlies the approach of treating patients with low dose IL2 to preferentially expand Treg with minimal impacts on effector T cells or other IL2R expressing cells (e.g. eosinophils) (Klatzmann and Abbas, 2015). Experimental studies characterized that low dose IL2, or IL2 in complex with an anti-IL2 antibody to enhance stability, preferentially expands Treg (reviewed in Pham *et al*., 2016). This therapeutic strategy holds promise in reducing many T cell mediated pathologies, or where an increased Treg response can limit inflammation-driven damage. In human clinical studies, low dose IL2 has been tested against type-1 diabetes, systemic lupus erythromatosus, and hepatitis C virus-induced vasculitis (Klatzmann and Abbas, 2015; Pham *et al*., 2016). One ‘side effect’ of this strategy is that IL2 may also expand $CD25^+$ innate lymphoid cells. The role of these cells in atherosclerosis is just beginning to be investigated (Engelbertsen *et al*., 2015). Furthermore, there is incomplete

knowledge on how IL2 therapy impacts on the emerging plasticity of Treg (Butcher *et al*., 2016).

Costimulatory Pathway Targeting Therapies

As an alternative to boosting Treg, therapies directly blocking effector T cell development or function could be developed to neutralise pathogenic T cell responses in atherosclerosis. Two prominent costimulatory pathways that inhibit or promote effector T cell responses are programmed cell death (PD-1) and Inducible T cell Costimulator (ICOS), respectively. Programmed cell death-1 (PD-1) and its ligands PD-L1 and PD-L2 are important in restraining pathogenic T cell responses. High PD-1 expression on T cells can also be associated with anergic or exhausted T cells in chronic inflammation in humans (McKinney *et al*., 2015) and may mark a pathogenic subset of T cells refractory to normal inhibitory signals. In murine atherosclerosis, PD-1 is associated with a protective role. *Ldlr*$^{-/-}$ mice lacking both PD-L1 and 2 develop enhanced systemic T cell activation, serum TNF and increased atherosclerosis (Gotsman *et al*., 2007). *Pd1*$^{-/-}$ mice also develop enhanced atherosclerosis (Bu *et al*., 2011; Cochain *et al*., 2014). In addition to systemic T cell activation, this was associated with increased apoptotic cell (both SMC and immune cells) accumulation within atherosclerotic plaques. Surprisingly, this result was not replicated in bone marrow chimeras with PD-L1/2 deficiency (Bu *et al*., 2011). Anti-PD1 antibody did not increase atherosclerotic size over a 3 week treatment but greatly increased T cell infiltration (Bu *et al*., 2011). Promoting PD-1 mediated immune restraint could therefore be beneficial in cardiovascular disease, in contrast to cancers where removing this inhibitory pathway enables anti-tumour T cell responses.

ICOS and its ligand ICOSL are members of the B7 family and are very important in promoting T helper cell responses, including Tregs. Deletion of ICOS in bone marrow cells reduced Tregs and increased atherosclerosis in *Ldlr*$^{-/-}$ mice (Gotsman *et al*., 2006). ICOS-Fc treatment also led to increased atherosclerosis, although a significant level of anti-mouse ICOS antibodies was induced by the treatment (Afek *et al*., 2005). More recently, targeting ICOS-ICOSL was effective in reducing atherosclerosis in a mouse model of combined autoimmunity and atherosclerosis (Clement *et al*., 2014). Deficiency in CD8^{+} regulatory T cells led to a prominent expansion of CD4^{+} T cell activation, particularly B helper Tfh. ICOS-ICOSL interactions are crucial in B2 cell-Tfh interactions, and anti-ICOSL treatment reduced Tfh cell levels and atherosclerosis. The role of Tfh-mediated responses and effects of

anti-ICOSL therapies in other models of atherosclerosis remain to be determined. A number of other costimulatory pathways have been associated with atherosclerosis in experimental models but progress towards the clinic is less advanced. For example, OX40 – OX40 ligand interactions promote T cell responses through stimulating both survival and expansion. In a regression model, where switching from western to chow diet (10 weeks of each) induces little change in plaque size but a very significant reduction in macrophages and expansion of collagen, anti-OX40 ligand induced plaque regression (Foks *et al*., 2013). Information on the impact of immunotherapies on cardiovascular parameters is constantly emerging, and this information is important in initiating novel strategies to directly counteract atherosclerosis progression.

B Cell Depletion

B cell depletion (BCD) therapy uses monoclonal antibodies against B cell-specific cell surface molecules such as CD20 or survival factors such as BAFF. BCD was developed against B cell derived cancers and is also now approved for use against autoimmune diseases. Although autoantibody production may also be modulated, impacts on effector/memory T cells may be the primary mode of action for B cell depletion (Lykken *et al*., 2014). Some human data on the impacts of B cell depletion on cardiovascular disease in autoimmune disease patients is beginning to emerge. Novikova *et al*. (2015) detected a reduced cIMT in rituximab treated individuals while Provan *et al*. (2015) found that after 12 months rituximab, pulse wave velocity, a measure of arterial stiffness, was reduced. A previous study (Raterman *et al*., 2013) found no impact on 6 months follow up. In atherosclerosis mouse models, primary studies showed reduced atherosclerosis when anti-CD20 treatment was administered prior to atherosclerotic development (Ait-Oufella *et al*., 2010; Kyaw *et al*., 2010). It was also effective treating progression of pre-established atherosclerosis (Kyaw *et al*., 2010), with further mechanistic insight into how B cell-T cell interactions regulate atherosclerosis still required. In myocardial infarction (MI) models, B cell depletion was also protective but through a distinct mechanism. B cells producing CCL7 (MCP-3) are critical in recruiting destructive monocytes to the infarction site and thus B cell depletion may represent an acute strategy post-MI in reducing infarct inflammation (Zouggari *et al*., 2013). We believe that current broad targeting of B cells using depletion therapies may be more practical for the acute treatment of ischemic heart disease post-MI rather than for the long-term treatment of atherosclerosis, however more targeted strategies with similar mechanisms may well be suitable. Several anti-BAFF monoclonal antibodies, Belimumab, Tabalumab and

Blisibimod, are in use in the clinic against autoimmune diseases and chronic lymphocytic leukaemia. In contrast to B cell depleting antibodies targeting B cell surface molecules (CD19, CD20, CD22), anti-BAFF therapies will also block other BAFF functions and so may have distinct modes of action. For example, BAFF may act directly on activated T cells (Scapini *et al*., 2010) and the BAFF receptor TACI is reported to be expressed on macrophages in mice (Allman *et al*., 2015). BAFF may also have impacts on cholesterol metabolism and adipose tissue function (Jackson *et al*., 2016; Kim and Hyun, 2016). Antibodies against the BAFF receptor target more specifically the pathogenic B2 cell subset and are effective in mouse models (Kyaw *et al*., 2013b) but are not yet translated to humans. To date there has been no studies investigating cardiovascular parameters in patients treated with BAFF-targeting therapies.

Immunization and Tolerization

In most cases, immunization and tolerization against a particular antigen should result in opposite responses in the host. In the case of modified LDL, studies in rabbits and mice have shown that both immunization (injection of antigen with adjuvants), and tolerization (non-adjuvant administration of peptides via oral or subcutaneous routes) can significantly reduce atherosclerosis (Palinski *et al*., 1995; Ameli *et al*., 1996; George *et al*., 1998). The target of tolerization is intentionally the regulatory T cell response, whereas immunization increases both innate and adaptive B cell responses, as well as regulatory T cell responses. The increased regulatory T cell response is proposed to be most active in reducing atherosclerosis, and may be the reason why both immunization and tolerization can protect from atherosclerotic development (Freigang *et al*., 1998), with increased Il5 that boosts innate B1 cell responses also a functional protective pathway (Binder *et al*., 2004). Complexing the PC antigen into liposomes was also effective in reducing atherosclerosis (Hosseini *et al*., 2015). Immunization with human ApoB100 induced a Th2 response and an IgG1 antibody response to both human and mouse LDL, but did not affect atherosclerosis (Engelbertsen *et al*., 2014). An alternative target of such adaptive immune-targeted strategies is hsp60/65, a known target of pathogenic T cell responses in mice (Zhong *et al*., 2016). Others include β2-GPI (George *et al*., 2004) and modified fibronectin (Dunér *et al*., 2011). Interestingly, different peptides from hsp60 had opposite effects on T cell driven immune responses and on atherosclerosis (Grundtman *et al*., 2015), suggesting the peptide itself as well as the stimulatory profile of the APC and the environment, can modulate outcomes. The development of peptides promoting a tolerogenic (anti-atherogenic) response could form the basis for future targeted therapies for

cardiovascular disease. Similarly, a study in mice demonstrated the protective effect of immunization with Streptococcus pneumonia polysaccharides, which contain PC epitopes (Binder *et al*., 2003). In humans, combined analysis of 8 observational studies suggest an association with pneumococcal polysaccharide vaccination and decreased risk of acute cardiovascular events in the older population (Ren *et al*., 2016). Recently, the AUSPICE trial was initiated to directly investigate the potential of this strategy (Ren *et al*., 2016).

The same IgM-targeted epitopes of oxLDL (MDA, PC) are also present on apoptotic cells. Apoptotic cell treatment was successful in inducing a strong IgG anti-PC response as well as boosting anti-PC IgM levels, leading to reduced atherosclerosis (Grasset *et al*., 2015). This led to a decrease in circulating cholesterol through an as yet ill-defined mechanism. Thus, antibody responses could directly modulate atherosclerosis at the level of circulating cholesterol upstream of vessel accumulation and oxidation. However, previous studies of lymphocyte deficient mice (Sage *et al*., 2014), and mice in which regulatory T cells are depleted also show significant changes in circulating cholesterol levels (Klingenberg *et al*., 2013). Impacts of modulation of dendritic cell levels, the archetypal T cell stimulating APC, on cholesterol have also complicated interpretation of those studies in understanding the importance of adaptive immune reactions in atherosclerosis (Gautier *et al*., 2009). Conversely, direct modulation of cholesterol in *Apoe*$^{-/-}$ mice can modulate CD4^{+} T cell responses (Chyu *et al*., 2014). Vaccination for atherosclerosis is an appealing concept and the integration of new knowledge both from ongoing human trials and from advanced interventions in mouse models will allow enhanced insight into the most effective mechanism to boost protective adaptive immunity.

Conclusion

Atherosclerosis is impacted at multiple stages by adaptive immune responses, providing multiple opportunities to target these responses to reduce disease progression. Protective influences defective in diseased individuals include humoral responses to modified LDL and regulatory T cell responses. There are diverse strategies in development to boost these pathways in humans. Alternative strategies to neutralise pro-inflammatory adaptive responses include blocking critical costimulatory pathways or other indirect anti-inflammatory treatments such as B cell depletion. Entering the translation phase is an exciting and promising prospect but issues include showing efficacy above existing therapies, such as statins, which may also have direct anti-inflammatory effects on top of lipid-lowering. In

addition, our knowledge of the network interactions within the immune system, and crosstalk with metabolic homeostasis must improve to develop accurately targeted treatments.

Acknowledgements

A.S. and Z.M. are supported by grants from the British Heart Foundation. Z.M. is also supported by FP7 VIA project and by Institut National de la Santé et de la Recherche Médicale (INSERM).

Author contributions

A.S. performed literature searches, designed the structure and prepared draft versions; A.S. and Z.M. wrote and prepared the final manuscript.

Competing interests

None

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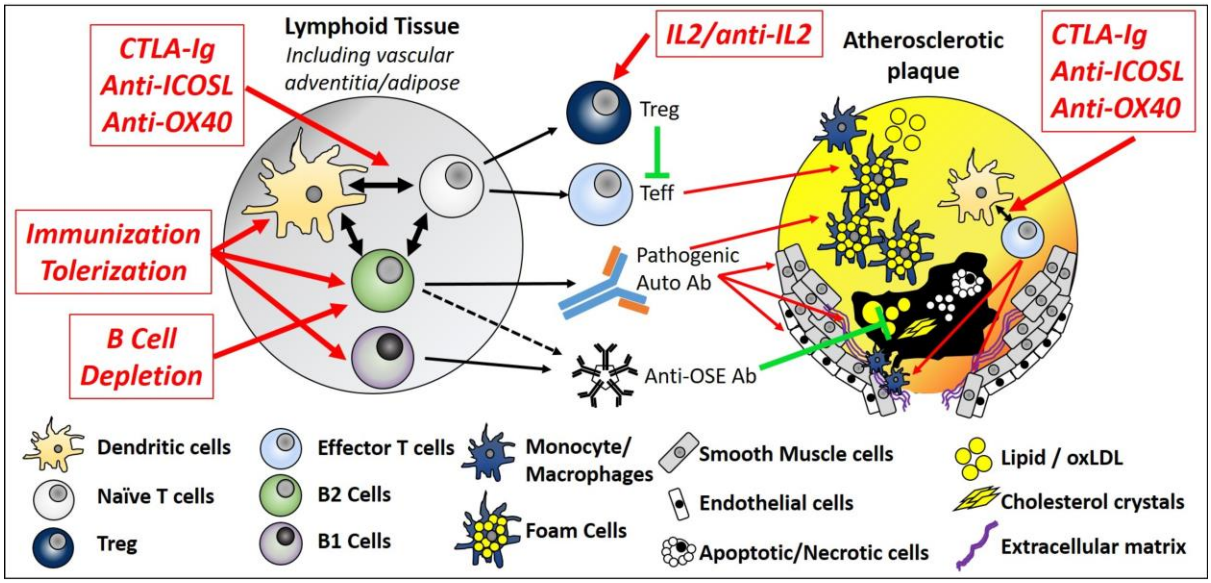
Figure Legends

Figure 1. Potential Therapeutic Strategies Targeting Adaptive Immunity in Atherosclerosis

Adaptive immune responses initiate in lymphoid organs (spleen, lymph nodes and in atherosclerosis in inflamed adventitia). Cell-cell interactions between dendritic cells, naïve T cells and B2 cells, dependent on costimulatory molecules and cytokines, result in atherogenic responses such as effector T cells and pathogenic autoantibodies from B2 cells but also protective responses such as regulatory T cells (Treg). B1 cells (and B2 cells) are stimulated by antigen and cytokines such as IL5 to produce atheroprotective anti-oxidation specific epitope (OSE) antibodies. Atherosclerosis is driven by macrophage foam cell formation leading to necrotic core formation. Rupture of the fibrous cap of smooth muscle cells and collagen matrix is a key therapeutic target. Pathogenic T cells (e.g. Th1), antagonised by Treg, promote inflammation and cell death of endothelial cells, smooth muscle cells and macrophages. Pathogenic autoantibodies, that bind plaque cells or extracellular matrix, may also promote inflammatory activation of vascular and immune cells. Protective antibodies neutralize and promote uptake of apoptotic/necrotic cells, modified lipids and microparticles. The point of intervention for potential therapeutic strategies (red boxes) are indicated.


CTLA-Ig
Anti-ICOSL
Anti-OX40
Lymphoid Tissue
Including vascular adventitia/adipose
IL2/anti-IL2
Atherosclerotic plaque
CTLA-Ig
Anti-ICOSL
Anti-OX40
Treg
Teff
Immunization
Tolerization
Pathogenic Auto Ab
B Cell Depletion
Anti-OSE Ab
Dendritic cells
Naïve T cells
Treg
Effector T cells
B2 Cells
B1 Cells
Monocyte/ Macrophages
Foam Cells
Smooth Muscle cells
Endothelial cells
Apoptotic/Necrotic cells
Lipid / oxLDL
Cholesterol crystals
Extracellular matrix